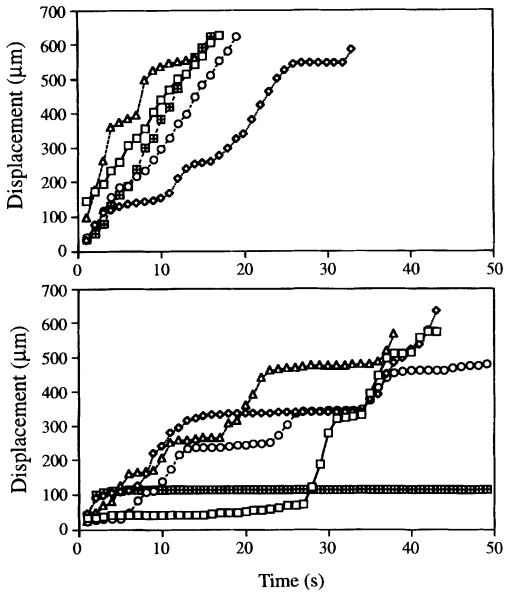

FIG. 4 Single-cell analysis of neutrophil rolling. Neutrophils were treated with DMSO carrier (upper panel) or with 25 μg ml^{-1} of the hydroxamic acid-based compound (lower panel) and then allowed to establish a rolling interaction at a wall shear stress of 3 dynes cm^{-2} on a MECA-79 antigen-coated borosilicate glass capillary tube. The rolling profile of distance (μm) versus time (s) was determined for 5 randomly selected cells in each group. Representative results from one of five independent experiments are shown.

accumulation under flow[18] and neutrophil aggregation[19]. In contrast, the presence of an appropriate chemokine signal results in global shedding of L-selectin and engagement of the CD18 integrins, leading to transendothelial migration. Global shedding of L-selectin may aid transmigration or may be a protective mechanism to prevent further trafficking of activated neutrophils. Thus L-selectin shedding may occur through two mechanisms–receptor ligation and chemokine activation–with distinct physiological outcomes.

L-selectin is part of an emerging and diverse class of proteins, including tumour necrosis factor (TNF)[20–22], transforming growth factor-α (TGF-α)[23], β-amyloid precursor protein[24], angiotensin-converting enzyme[25], and interleukin-6R[26], whose cell-surface expression is regulated by activation-induced proteolysis at a membrane proximal site. Recently hydroxamic acid-based peptides have been demonstrated to inhibit activation-induced shedding of TNF[20–22] and TNF receptor[27], suggesting that a related protease activity may be involved in the regulation of at least TNF and L-selectin. Both the membrane-bound and soluble forms of TNF and other receptors and growth factors are thought to be biologically active[28,29]. The receptor ligation-induced shedding mechanism shown for L-selectin may have implications for these other biological systems as a cell contact-dependent, activation-independent means of rapidly processing growth factors, cytokines and receptors presented on the cell surface.

A shear threshold requirement for L-selectin-mediated adhesion has recently been described, and it has been speculated that the shear threshold may prevent adhesion under low flow conditions[32]. The shear threshold requirement and the ligation-dependent shedding of L-selectin are not mutually exclusive models for regulating neutrophil aggregation and accumulation. □

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Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis

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BINDING of Fas ligand or an agonistic anti-Fas antibody induces apoptosis in Fas-bearing cells[1]. The interleukin-1β-converting enzyme (ICE) is a cysteine protease[2] that is involved in apoptosis induced by various stimuli, including Fas-mediated apoptosis[3–8]. Several ICE homologues have been identified, and these are subdivided into three groups (ICE-, CPP32- and Ich-1-like proteases)[9–18]. We show here that specific inhibitors of ICE- or CPP32-like proteases can inhibit Fas-mediated apoptosis. Transient ICE-like activity was found in the cytosolic fraction of Fas-activated cells, whereas ICE-dependent, CPP32-like activity gradually accumulated in the cytosol. Cell lysates from mouse lymphoma supplemented with either recombinant ICE or CPP32 induced apoptosis of nuclei. The CPP32 inhibitor inhibited ICE- or CPP32-induced apoptosis in the cell-free system, whereas the ICE-inhibitor only inhibited ICE-induced apoptosis. Cell extracts from thymocytes from ICE-null mice induced apoptosis in the cell-free system when it was supplemented with CPP32. These results indicate that Fas sequentially activates ICE- and CPP32-like proteases, and that downstream CPP32, together with a component(s) in the cytoplasm, causes apoptosis of nuclei.

Among members of the ICE family, ICE and CPP32 have different substrate specificities[16,19]. We examined the involvement of ICE and CPP32 in Fas-mediated apoptosis using two tetrapeptides: acetyl-Tyr-Val-Ala-Asp-CHO (Ac-YVAD·cho) and

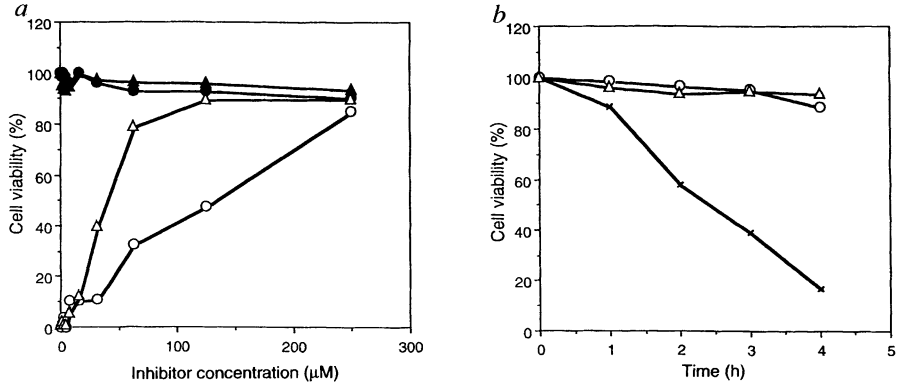

FIG. 1 Effect of Ac-YVAD·cho or Ac-DEVD·cho on Fas-mediated apoptosis. *a*, Mouse W4 cells[30] (2.5×10^4 cells) in 0.1 ml were preincubated for 1 h with various concentrations of either Ac-YVAD·cho (triangles) or Ac-DEVD·cho (circles) (Peptides Inst. Osaka). Cells were then incubated at 37 °C for 4 h in the presence (open symbols) or absence (filled symbols) of 300 ng ml^{-1} anti-Fas antibody (Jo2) (ref. 30). Percentages of viable cells were determined by Trypan-blue exclusion[24]. *b*, After preincubation at 37 °C for 1 h without (crosses) or with 300 μM Ac-YVAD·cho (triangles) or Ac-DEVD·cho (circles), W4 cells (2.5×10^4 cells) were incubated at 37 °C for various periods in the presence of 300 ng ml^{-1} anti-Fas antibody. Cell viability was determined as for *a*.

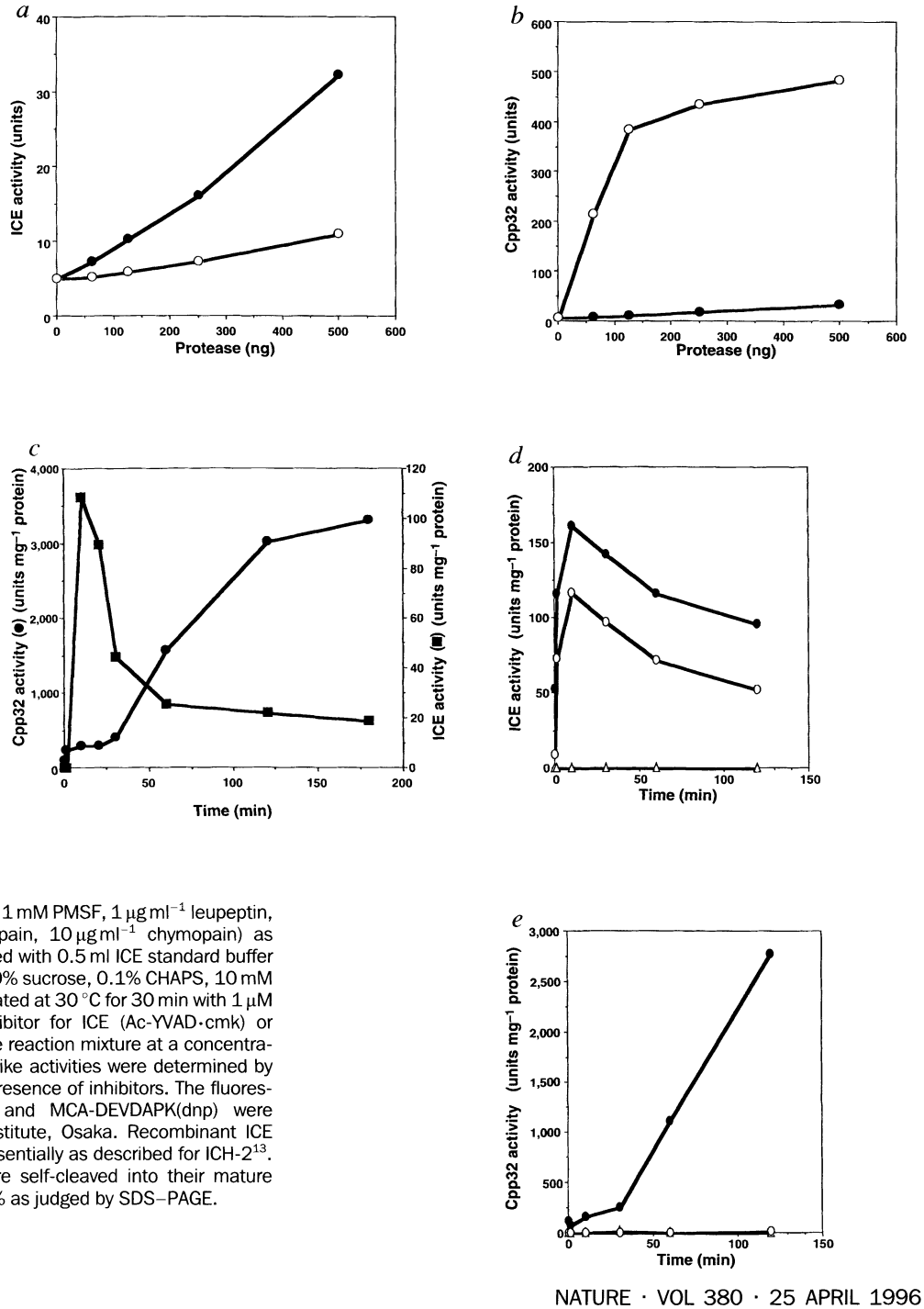

FIG. 2 Sequential activation of ICE-like and CPP32-like proteases during Fas-induced apoptosis. *a* and *b*, The fluorescent substrate, either MCA-YVADAPK(dnp) (*a*), or MCA-DEVDAPK(dnp) (*b*), was incubated at 30 °C for 30 min with various amounts of the recombinant ICE (filled circles) or CPP32β (open circles), and the fluorescence of the cleaved substrates was determined using a spectrofluorometer set at an excitation wavelength of 325 nm and an emission wavelength of 392 nm. *c*, Cytosolic extracts were prepared from W4 cells (1×10^7 cells) which were treated at 37 °C with 300 ng ml^{-1} of anti-Fas antibody for various times. ICE- and CPP32-like activity in the lysates (36 μg protein) was then determined using the fluorescent substrates, MCA-YVADAPK(dnp) (squares) or MCA-DEVDAPK(dnp) (circles), respectively. *d* and *e*, W4 cells were treated for various times with anti-Fas antibody alone (filled circles) or in the presence of either 300 μM Ac-YVAD·cmk (open triangles) or Ac-DEVD·cho (open circles). The ICE-like (*d*) or CPP32-like activity (e) in the cell extract (36 μg protein) was assayed; one unit corresponds to the activity that cleaves 1 pmol of the respective fluorescent substrate at 30 °C in 30 min.

METHODS. Cytosolic extracts were prepared by repeated freezing and thawing of cells in 100 μl extraction buffer (50 mM PIPES-NaOH, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 20 μM cytochalasin B, 1 mM PMSF, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 50 μg ml^{-1} antipain, 10 μg ml^{-1} chymopain) as described[21]. Cell lysates were then diluted with 0.5 ml ICE standard buffer (100 mM HEPES-KOH buffer, pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT, 0.1 mg ml^{-1} ovalbumin), and incubated at 30 °C for 30 min with 1 μM fluorescent substrate. The specific inhibitor for ICE (Ac-YVAD·cmk) or CPP32 (Ac-DEVD·cho) was added to the reaction mixture at a concentration of 1 μM. Specific ICE- and CPP32-like activities were determined by subtracting the values obtained in the presence of inhibitors. The fluorescent substrates, MCA-YVADAPK(dnp) and MCA-DEVDAPK(dnp) were custom-synthesized at the Peptides Institute, Osaka. Recombinant ICE and CPP32β were prepared in *E. coli* essentially as described for ICH-2[13]. The recombinant ICE and CPP32β were self-cleaved into their mature forms; their purities were more than 80% as judged by SDS–PAGE.

acetyl-Asp-Glu-Val-Asp-CHO (Ac-DEVD·cho), which are specific inhibitors of ICE and CPP32, respectively[16,19]. As shown in Fig. 1, mouse WR19L transformant cells (W4), which constitutively express mouse Fas, were killed within four hours by an agonistic anti-Fas antibody. When W4 cells were preincubated with either Ac-YVAD·cho or Ac-DEVD·cho, Fas-induced apoptosis was inhibited. Kinetic analysis of the death process in the presence of Ac-YVAD·cho or Ac-DEVD·cho confirmed this inhibitory effect (Fig. 1*b*).

The ICE- and CPP32-like activities in Fas-activated cell extracts were then measured. To distinguish between the two activities, we prepared two peptides, MCA-YVADAPK(dnp) and MCA-DEVDAPK(dnp), in which specific peptides were coupled to the highly fluorescent (7-methoxycoumarin-4-yl)acetyl group (MCA) and its quenching 2,4-dinitrophenyl (dnp) group. As shown in Fig. 2*a*, recombinant ICE cleaved MCA-YVADAPK(dnp), but DPP32β was only very weakly active on the substrate. On the other hand, CPP32β efficiently cleaved MCA-DEVDAPK(dnp), whereas ICE had little activity on this substrate (Fig. 2*b*). The specific activities of the recombinant ICE for MCA-YVADAPK(dnp) and CPP32 for MCA-DEVDAPK(dnp) were 64 and 3,300 units per μg protein, respectively.

W4 cells were treated for various periods with the anti-Fas antibody, after which the ICE- and CPP32-like activities in the cytosolic extracts were determined using fluorescent substrates. Extracts from untreated W4 cells did not show ICE-like activity (Fig. 2*c*). Fas activation induced transient ICE-like activity with a peak at 10 min (110 units per mg protein). The cytosol of untreated W4 cells showed a low level of CPP32-like activity, which was gradually increased up to 3,300 units per mg protein by Fas activation. If we assume that the ICE- and CPP32-like proteases in the cytosol have specific activities similar to recombinant ICE and CPP32, the results in Fig. 2*c* suggest that ICE- and CPP32-like proteases were comparably activated but with different kinetics during Fas-mediated apoptosis. The appearance of ICE-like activity before CPP32-like activity suggested that the activation of the CPP32-like protease might depend on the presence of the ICE-like protease. To examine this possibility, we treated W4 cells with anti-Fas antibody in the presence of Ac-YVAD·chloromechylketone (cmk) or Ac-DEVD·cho, and determined the ICE- and CPP32-like activities in the cytosol. We found that Ac-YVAD·cmk completely inhibited the generation of ICE-like activity (Fig. 2*d*), but that Ac-DEVD·cho had only a small effect on it. In contrast, both Ac-YVAD·cmk and Ac-DEVD·cho completely inhibited Fas-induced generation of CPP32-like activity (Fig. 2*e*). These results indicate that the production of CPP32-like activity during Fas-mediated apoptosis depends on the previous presence of ICE-like activity.

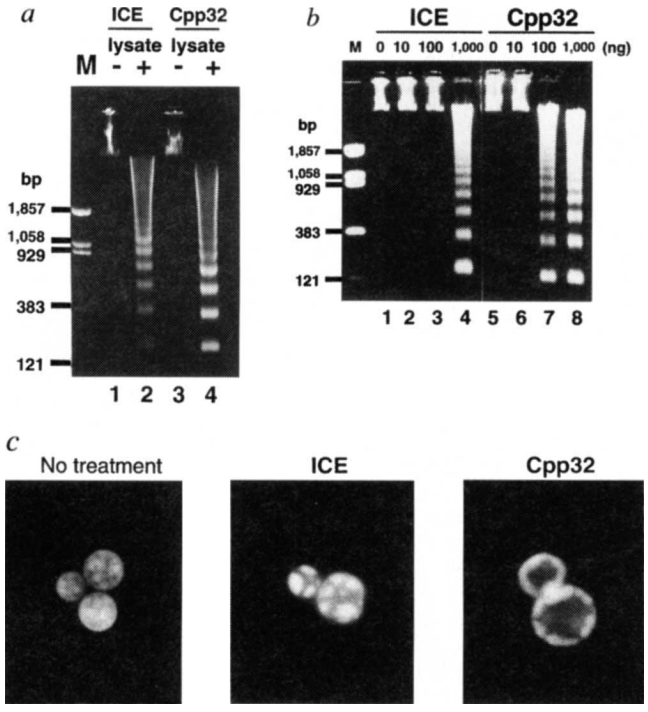

FIG. 3 Apoptosis induced by recombinant ICE or CPP32β. *a*, Nuclei from mouse liver[21] were incubated with recombinant human ICE (3 μg) or CPP32β (100 ng) in the absence or presence of cytosolic extracts (140 μg) from unstimulated W4 cells. After incubation, nuclei were collected by centrifugation, and the chromosomal DNA analysed by electrophoresis on a 1.5% agarose gel. *b*, Nuclei were incubated with the indicated amounts of either ICE or CPP32β combined with the cell lysates (140 μg) from unstimulated W4 cells and the chromosomal DNA analysed. *c*, Nuclei from mouse liver were incubated with extracts (140 μg) from unstimulated W4 cells (left panel), or with cell extracts supplemented with either 3 μg recombinant ICE (middle) or 200 ng of CPP32β (right). After incubation, nuclei were stained with 10 μg ml^{-1} of 4′,6′-diamidino-2-phenylindole (DAPI), and observed under a fluorescence microscope with a UV-2A combination filter (Nikon OPTIPHOT).

METHODS. *In vitro* apoptosis was carried out essentially as described[21]. In brief, 3×10^6 nuclei from mouse liver were incubated at 37 °C for 3 h in 27 μl of R buffer (10 mM HEPES-KOH, pH 7.0, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 2 mM ATP, 10 mM creatine phosphate, 50 μg ml^{-1} creatine kinase and 0.2 mg ml^{-1} BSA).

We next investigated whether ICE-induced apoptosis in a cell-free system is mediated by CPP32-like activity. As shown in Fig. 4, when the lysates from W4 cells were incubated with ICE, CPP32-like activity was generated in the lysates (lane 1). Ac-YVAD·cho inhibited ICE activity, the production of CPP32-like protease in the cell lysates, and ICE-induced apoptosis of nuclei (lane 2). On the other hand, Ac-DEVD·cho did not inhibit ICE, but did inhibit ICE-induced production of CPP32-like activity in the cell lysates and apoptosis in the nuclei (lane 3). CPP32β did not generate ICE-like activity (lane 4), and CPP32-induced apoptosis was inhibited by Ac-DEVD·cho but not Ac-YVAD·cho (lanes 5 and 6). These results suggest that ICE first activates CPP32-like protease in the cell lysates, and that CPP32-like protease then causes apoptosis in nuclei together with a component(s) in the lysates. Thymocytes from mice lacking ICE are resistant against Fas-mediated apoptosis[8]. When cell lysates of thymocytes from ICE-null mice[23] were supplemented with recombinant CPP32β, they induced apoptosis in nuclei as efficiently as lysates from wild-type mice (Fig. 4*d*). These results confirmed that CPP32-induced apoptosis does not require ICE, and suggested that the defect in Fas-mediated apoptosis of thymocytes in ICE-null mice is due to the failure of ICE-dependent activation of CPP32.

Three models may explain the contribution of at least seven ICE members in apoptosis: (1) each member independently induces apoptosis; (2) members of the family sequentially activate other members; or (3) at least two members are required in parallel to induce apoptosis. Here we have shown that Fas induces a sequential activation of ICE-like and CPP32-like proteases, and

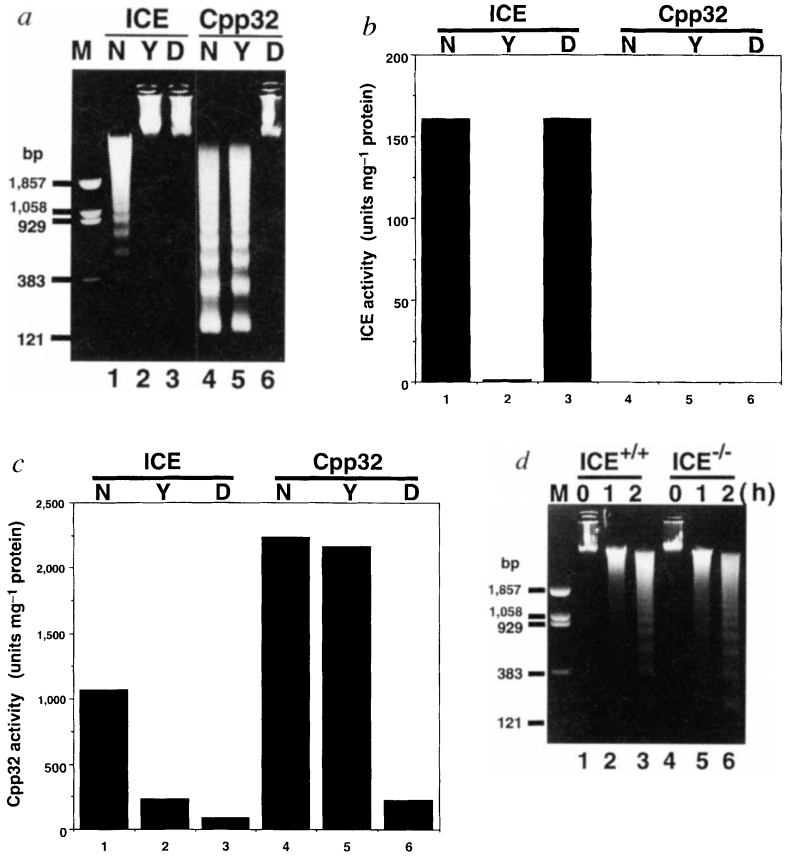

FIG. 4 CPP32β-induced apoptosis does not apparently require ICE. *a*–*c*, Cell lysates from unstimulated W4 cells (140 μg) were incubated at room temperature for 15 min with no additions (N) (lanes 1 and 4) or in the presence of either 180 nM Ac-YVAD·cho (Y) (lanes 2 and 5) or 72 nM Ac-DEVD·cho (D) (lanes 3 and 6) in 24 μl of R buffer. Recombinant ICE (2.5 μg) (lanes 1–3) or CPP32β (200 ng) (lanes 4–6) was added to the reaction mixture and incubated at room temperatures for 15 min. Nuclei (3×10^6 in 3 μl) were then added to the mixture, and further incubated at 37 °C for 3 h. Using 6-μl aliquots, chromosomal DNA from the nuclei was analysed by electrophoresis on a 1.5% agarose gel (*a*), 2-μl aliquots were used to assay ICE-like (*b*) and CPP32-like (*c*) activities with 1 μM MCA-YVADAPK(dnp) or MCA-DEVDAPK(dnp) as substrate. *d*, Cell lysates were prepared from wild-type ($ICE^{+/+}$) or ICE-null mice ($ICE^{-/-}$) as described[21]. Nuclei (3×10^6) were incubated for the indicated periods at 37 °C in a final volume of 30 μl with the cell lysates (25 μg), which were supplemented with 20 ng recombinant CPP32β. Chromosomal DNA was analysed as for *a*.

that downstream CPP32 is sufficient to cause apoptotic DNA degradation in nuclei together with a component(s) in the cytoplasm. These results support the second model. However the ICE-like proteases seem to be redundant, because, although the thymocytes from ICE-null mice show defective Fas-mediated apoptosis[8], the mice did not show the lymphoproliferation phenotype seen in mice carrying the loss-of-function mutation of Fas or Fas ligand[1]. Whether or not CPP32-like activity is also mediated by redundant proteases remains to be investigated.

Fas-mediated apoptosis proceeds without RNA or protein synthesis[24–26], indicating that the appearance of ICE- or CPP32-like activity is a post-translational activation of these proteases. It is likely that ICE-like and CPP32-like proteases are sequentially cleaved to become active forms. If so, why is the ICE-like protease only transiently activated? It is possible that mammalian cells contain CrmA- or p35-like inhibitors of ICE[27,28]. The signal from Fas may transiently stimulate the dissociation of such inhibitors from the mature ICE-like protease or, alternatively, ICE activation might be followed by the activation of a different protease that cleaves ICE in order to inactivate it. CPP32-like activity gradually accumulates in the cytosol of Fas-activated cells. Although CPP32 can be directly cleaved by ICE[17], it is not yet clear whether the ICE-like protease directly cleaves the CPP32-like protease, or whether other proteins acting between the two could be involved during Fas-induced apoptosis. Active CPP32 alone could not induce apoptosis in a cell-free system, but it caused apoptosis in nuclei supplemented with cell lysates. The requirement of the cytoplasmic fraction to induce apoptosis suggests that one or more additional components in the cytoplasm help CPP32 to enter the nucleus, where it cleaves various substrates such as poly(ADP) ribose polymerase[29]. But it is also possible that the actual substrate for CPP32 that causes DNA degradation is in the cytoplasm, and that the activated substrate goes into the nucleus. In any event, our *in vitro* system of apoptosis using recombinant ICE or CPP32 will be useful in determining the apoptotic pathway downstream of CPP32. □

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